Brief Genetics Report

Analysis of the Coding and Promoter Regions of the Autoantigen IA-2 in Subjects With and Without Autoantibodies to IA-2

Tao Cai,[1] Jingping Xie,[1] Jin-Xiong She,[2] and Abner Louis Notkins[1]

Despite extensive studies on HLA polymorphism, there have been few, if any, studies on allelic forms or mutations in proteins that serve as autoantigens. The present experiments were designed to look for alterations in the coding and promoter regions of the autoantigen IA-2 in type one (insulin-dependent) diabetic patients with autoantibodies to IA-2 as compared with siblings without diabetes or autoantibodies to IA-2. Genomic DNA was used as a template and was amplified by polymerase chain reaction, with pairs of primers encompassing the promoter region and the 23 exons of the coding region of IA-2. A total of nine nucleotide changes were found in the coding region of the six type 1 diabetic patients; four were silent and five were missense changes, but all occurred in the extracellular domain of IA-2 to which autoantibodies are not directed. Few, if any, changes were found in the 5′ upstream (−706 to +135) promoter region. The results of the experiments support the null hypothesis that differences among individuals in the nucleotide and amino acid sequences of the promoter and coding regions of IA-2, respectively, do not account for why some individuals develop autoantibodies to IA-2 and others do not. *Diabetes* 50:2406–2409, 2001

The immune response to an antigen requires the processing of that antigen into peptides. Processed peptides then bind to class I or II HLA molecules, and the complexes are presented to receptors on T-cells (1). The magnitude of the immune response is a function of antigen presentation, which in turn is dependent on antigen-binding to specific HLA molecules. Which peptides bind is defined by the amino acid sequence within the binding groove of the HLA molecule (2). The diversity of HLA alleles ensures a broad spectrum of immune responses within the population.

Certain autoimmune diseases are known to be strongly linked to the HLA haplotype of an individual (3). One of the strongest linkages is with type 1 (insulin-dependent) diabetes (4,5). Individuals who are of the HLA-DQ and/or DR haplotype (i.e., the DRB1*0301, DRB1*0401, DQA1*0301, and/or DQB1*0302 alleles) are at a considerably higher risk of developing type 1 diabetes than individuals with other haplotypes, and >90% of type 1 diabetic patients are of the HLA-DQ and/or DR haplotype (4,6,7). In contrast, individuals who carry the HLA allele DQB1*0602 are at a considerably lower risk of developing type 1 diabetes, and the DQB1*0602 allele may in fact be protective (8). Susceptibility to type 1 diabetes can be influenced by the presence or absence of a single amino acid within the HLA molecule. For example, resistance to type 1 diabetes is strongly associated with an aspartic acid at position 57 of the HLA-DQβ chain. When aspartic acid is not present at position 57, there is a significant increase in the risk of type 1 diabetes (9,10).

Despite extensive studies on HLA molecules, very little consideration has been given to the possibility that there might be distinct alleles encoding proteins that act as autoantigens, and that this could result in differences in the amino acid composition of the autoantigens. If such allelic differences exist, the processed autoantigenic peptides might fit better into specific HLA grooves and thereby make the triggering of an autoimmune response more likely. This might account for why some individuals develop autoantibodies to an antigen and others do not. Similarly, polymorphisms in the promoter region might result in aberrant expression of an autoantigen and thereby increase the likelihood of triggering an autoimmune response. Despite these possibilities, the nucleotide sequence of the coding and promoter regions of autoantigens in patients with and without autoantibodies has not been studied.

IA-2, a member of the protein tyrosine phosphatase family, is a major autoantigen in type 1 diabetes (11). Of all newly diagnosed type 1 diabetic patients, ~70% have autoantibodies to IA-2, and these autoantibodies appear years before the development of clinical disease. The presence of autoantibodies to both IA-2 and GAD are highly predictive markers for identifying individuals at risk of developing type 1 diabetes (12), and it is estimated that if both autoantibodies are present, the likelihood of developing type 1 diabetes within 5 years is >50%. The IA-2 molecule is 979 amino acids in length and consists of an intracellular, transmembrane, and extracellular domain.

From the [1]Experimental Medicine Section, Oral Infection and Immunity Branch, National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, Maryland; and the [2]Department of Pathology, University of Florida, Gainesville, Florida.

Address correspondence and reprint requests to Dr. Abner Louis Notkins, National Institutes of Health, Bldg. 30, Rm. 121, 30 Convent Dr. MSC 4322, Bethesda, MD 20892-4322. E-mail: anotkins@dir.nidcr.nih.gov.

Received for publication 5 October 2000 and accepted in revised form 8 July 2001.

PCR, polymerase chain reaction.

TABLE 1
Primers used for 23 exons, flanking intronic regions, and promoter region of human IA-2

Primers	Region	Forward primer (5′→3′)	Reverse primer (3′→5′)
I	Exon 1 and 5′-proximal	catgtagtatgcaccagtcc	agcccaagtttcttcctgac
II	Exon 2–3	acctttttcctctacagGCTGTC	aatctctctcctccagtgcacc
III	Exon 4–7	ctctttcctgatcagGATTG	cctctgcactggacacttacC
IV	Exon 8–12	ccacttgtgttgtgTAGACC	ctgatattccagttagcgca
V	Exon 13–18	ccttgattctagccttgttcc	ggccctgctgaccTCATATACG
VI	Exon 19	gcccctgccgggcagGTGAA	gcccatgcgg tccctccag
VII	Exon 20–23	gcccttctgtctctctccca	gtacacagagatgctcacacagg
A	Alu 1	cacatctaatgatgaggcca	ggactggtgcatacgaacat
B	Alu 2	ggcggtggttgttcttaattg	cctaagtgctgttatgacacc
C	Alu 3	acaacacttggctgggataacagg	tgtaatgacctctaacagtgactaatac
D	5′-distal	acaacacttggctgggataacagg	ggactggtgcatacgaacat

Primers were designed from genomic sequences deposited in GenBank (accession no. AF042285 and Q16849). Primers located in introns are lower-cased; primers located in exons are capitalized.

Autoantibodies are directed exclusively to the intracellular domain (13,14). Genomic structure and chromosome analysis revealed that IA-2 is located on chromosome 2q35 and consists of 23 exons that span ~20 kb (15). Although the amino acid sequence of IA-2 from nondiabetic subjects has been determined, there have been no studies comparing the IA-2 amino acid sequence of diabetic subjects who are IA-2 autoantibody–positive with nondiabetic subjects who are IA-2 autoantibody–negative. The present experiments were initiated to compare the nucleotide sequence of the coding and promoter regions of IA-2 in individuals who were autoantibody-positive and -negative. Because IA-2 mRNA is not expressed in lymphocytes or other easily accessible tissue, genomic DNA, extracted from peripheral lymphocytes, was used to determine nucleotide sequences.

To look for alterations in IA-2 that might account for the development of IA-2 autoantibodies, genomic DNA from six IA-2 autoantibody–positive type 1 diabetic patients was used as template. Seven pairs of primers covering the 23 exons of the coding region of IA-2 were prepared (Table 1 and Fig. 1) and used to amplify IA-2 genomic DNA by polymerase chain reaction (PCR). The size of the PCR products for each of the regions amplified (Fig. 1) corresponded to the expected sizes. The PCR products were then cloned, sequenced, and compared with the sequence of IA-2 from nondiabetic siblings and normal control data in the GenBank (accession no. AF042285 and Q16849). As seen in Table 2, a total of nine nucleotide substitutions were found, resulting in five amino acid changes. Seven of the nine nucleotide substitutions were found in different exons. In three of the patients, a nucleotide substitution was found in exon 5, but each was located at a different position, and two resulted in a conserved amino acid change (patient 3, $A^{169}\rightarrow V$; patient 5, $A^{152}\rightarrow V$). We examined the frequency of these three mutations in exon 5. The whole exon was amplified from the genomic DNA of 190 type 1 diabetic patients and 190 normal control subjects of Caucasian origin. Single-strand conformation polymor-

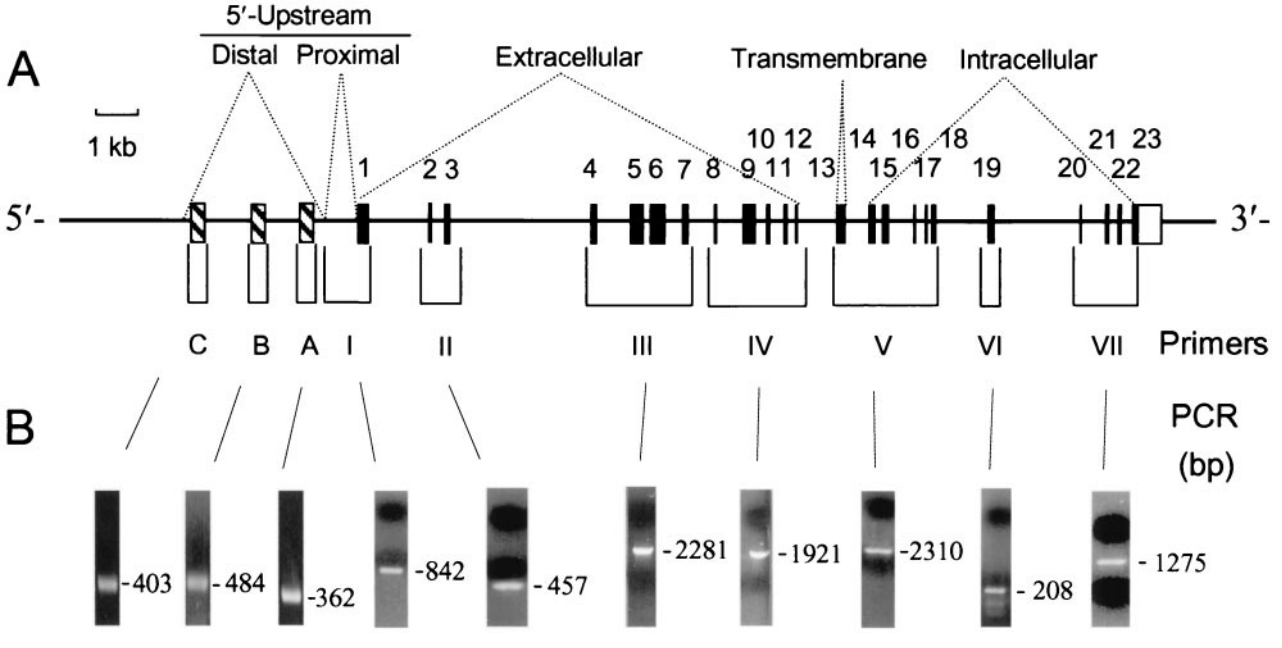

FIG. 1. PCR amplification of coding and promoter regions of human IA-2. *A*: Exons are represented by black vertical boxes and numbered 1–23. Introns are denoted by horizontal lines between exons. ▨, different *Alu* repeats in the distant 5′ region. Primer positions are indicated by roman numbers I-VII and the letters A, B, and C. Not shown is the paired primers designated D in Table 1 covering the region A–C (nucleotide numbers −688 to −3,091). *B*: PCR products analyzed on a 1% agarose gel.

TABLE 2
IA-2 sequence variations in IA-2 autoantibody–positive patients

Patient no.	Exons	Type of mutation	Nucleotide change	Amino acid change
1	3	Silent	$C^{298}\rightarrow T$	None
	7	Missense	$A^{1137}\rightarrow G$	$E^{137}\rightarrow G$
2	13	Silent	$G^{1921}\rightarrow A$	None
3	5	Missense	$C^{579}\rightarrow T$	$A^{169}\rightarrow V$
4	6	Missense	$T^{909}\rightarrow A$	$L^{279}\rightarrow H$
	18	Silent	$T^{2557}\rightarrow C$	None
5	4	Missense	$C^{441}\rightarrow A$	$P^{123}\rightarrow L$
	5	Missense	$C^{528}\rightarrow T$	$A^{152}\rightarrow V$
6	5	Silent	$T^{475}\rightarrow C$	None

phism analysis (16) did not identify any subjects with the same mutations, suggesting that these mutations have very low frequencies in the Caucasian population. Of the five amino acid changes, all occurred within the extracellular domain of IA-2 to which autoantibodies are not directed.

Recently, we determined the sequence of the proximal 5′ upstream region of IA-2 and showed that it possessed promoter activity (15). In the current study, primer 1, which encompasses this region (−706 nt to +135 nt, including exon 1), was used to amplify the genomic DNA of diabetic and nondiabetic subjects by PCR, and the PCR products were sequenced. The DNA from the six nondiabetic autoantibody-negative subjects revealed identical sequences (GenBank accession no. AF042258), but showed several nucleotide changes compared with the sequence originally reported (GenBank accession no. gi:2801767). The sequence of the DNA from the six diabetic autoantibody-positive subjects was identical to that of the nondiabetic autoantibody-negative subjects.

In the distal 5′ upstream region (−688 nt to −3,091 nt), using four pairs of primers (A–D) (Table 1 and Fig. 1), three *Alu* consensus sequences (i.e., A, B, and C) were found (GenBank accession no. AF042285) that belong to the *Alu* subfamily Sb (17). As expected, *Alu* polymorphisms were detected in both normal subjects and diabetic patients (Table 3), but the frequency is still not known, and the significance, if any, of these polymorphisms at such a long distance from the IA-2 promoter or coding regions is uncertain.

Mutations in the promoter region of specific genes or the transcription factors that interact with these genes can influence the time and magnitude of protein expression (18–20). In the present study, we failed to find any significant changes in the proximal 5′ upstream region of IA-2 in type 1 diabetic patients. This argues that alterations in the promoter region are not responsible for the aberrant expression of IA-2, which might trigger the development of IA-2 autoantibodies. The idea that alterations in transcription factors expressed in diabetes-prone individuals, but not in control subjects, also might affect the time and magnitude of IA-2 expression, remains to be investigated.

We conclude that neither allelic forms of IA-2 nor mutations in the coding or promoter regions of the IA-2 gene are responsible for triggering autoantibodies to IA-2. Thus, at least in the case of one autoimmune disease, type 1 diabetes, the results presented here support the null hypothesis that differences in the amino acid sequence of the autoantigen do not explain why some individuals develop autoimmune disease and others do not.

RESEARCH DESIGN AND METHODS

Subjects. Genomic DNA was obtained from six diabetic and six nondiabetic subjects (three male and three female subjects in each group) from the Human Biological Data Interchange (HBDI, Philadelphia, PA). The subjects came from families with two or more siblings with type 1 diabetes and at least one sibling without diabetes. The diabetic subjects ranged in age from 2 to 12 years, and five of the six that were genotyped were HLA-DRB1*0301 or 0401 or DQB1*0302. The genotypes of the nondiabetic siblings varied considerably, and were HLA DRB1*0701, *1501, *0401, and *0302 and DQB1*02, *0602, *0302, *0301, and *0402. All six diabetic patients, but none of the nondiabetic siblings, had autoantibodies to IA-2, as determined by radioimmune precipitation of recombinant IA-2 (21). For the single-strand conformation polymorphism studies, we used genomic DNA from 190 patients with type 1 diabetes and 190 unrelated control subjects of Caucasian origin collected from North-Central Florida (22).

Cloning and sequencing of coding and 5′ upstream regions of human IA-2. Paired primers were prepared as indicated in Table 1. Genomic DNA served as the template and was amplified by PCR. PCRs were carried out in 50 μl Buffer F (Epicentre, Madison, WI), 300 nmol/l of each primer, 2.5 units *AmpliTaqGold* (Perkin-Elmer, Wellesley, MA), and 100 ng of genomic DNA from patients or control subjects. Reactions were cycled in a PCR 9700 (PE Biosystems, Foster City, CA), with denaturation at 95°C for 10 min, followed by 32 cycles of denaturation at 94°C for 20 s, annealing at 60°C for 45 s, and extension at 72°C for 1 min, except for those of primers III-V extended at 72°C for 2 min. There was a final extension at 72°C for 10 min for all primers. PCR products were analyzed on 1% agarose gels, and bands were cut and cloned into PCR2.1 vector (Invitrogen, Carlsbad, CA). Three clones from each PCR were selected and sequenced from both orientations on a 373A DNA sequencer (Applied Biosystems, Foster City, CA). Sequence searches were performed using the BLAST and GAP program (Version 10; Genetics Computer Group, Madison, WI).

TABLE 3
Alu polymorphisms in the distal 5′-upstream region of IA-2 in IA-2 antibody–positive and –negative subjects

IA-2 Ab^+	Alu-repeat	Nucleotide change	IA-2 Ab^-	Alu-repeat	Nucleotide change
1	B	$C^{-1828}\rightarrow T$	1		None
2	B	Insert A^{-1562}	2	B	Insert A^{-1562}
3	A	$A^{-831}\rightarrow G$	3		None
4	B	Insert A^{-1562}	4		None
5	B	Insert A^{-1562}	5		None
6	A	$A^{-831}\rightarrow G$	6	A	$A^{-831}\rightarrow G$

REFERENCES

1. Margulies DH: The Major histocompatibility complex. In *Fundamental Immunogy*. Paul WE, Ed, 1999, Philadelphia, PA, Lippincot-Raven, p. 263–285
2. Fridkis-Hareli M, Rosloniec EF, Fugger L, Strominger JL: Synthetic amino acid copolymers that bind to HLA-DR proteins and inhibit type II collagen-reactive T cell clones. *Proc Natl Acad Sci U S A* 95:12528–12531, 1998
3. Nepom GT, Erlich H: MHC class-II molecules and autoimmunity. *Annu Rev Immunol* 9:493–525, 1991
4. She JX: Susceptibility to type I diabetes: HLA-DQ and DR revisited. *Immunol Today* 17:323–329, 1996
5. Herr M, Dudbridge F, Zavattari P, Cucca F, Guja C, March R, Campbell RD, Barnett AH, Bain SC, Todd JA, Koeleman BP: Evaluation of fine mapping strategies for a multifactorial disease locus: systematic linkage and association analysis of IDDM1 in the HLA region on chromosome 6p21. *Hum Mol Genet* 9:1291–1301, 2000
6. Nepom GT, Kwok WW: Molecular basis for HLA-DQ associations with IDDM. *Diabetes* 47:1177–1184, 1998
7. Kockum I, Sanjeevi CB, Eastman S, Landin-Olsson M, Dahlquist G, Lernmark A: Complex interaction between HLA DR and DQ in conferring risk for childhood type 1 diabetes. *Eur J Immunogenet* 26:361–372, 1999
8. Pugliese A, Gianani R, Moromisato R, Awdeh ZL, Alper CA, Erlich HA, Jackson RA, Eisenbarth GS: HLA-DQB1*0602 is associated with dominant protection from diabetes even among islet cell antibody–positive first-degree relatives of patients with IDDM. *Diabetes* 44:608–613, 1995
9. Todd JA, Bell JI, McDevitt HO: HLA-DQ beta gene contributes to susceptibility and resistance to insulin-dependent diabetes mellitus. *Nature* 329:599–604, 1987
10. Morel PA, Dorman JS, Todd JA, McDevitt HO, Trucco M: Aspartic acid at position 57 of the HLA-DQ beta chain protects against type I diabetes: a family study. *Proc Natl Acad Sci U S A* 85:8111–8115, 1988
11. Lan MS, Lu J, Goto Y, Notkins AL: Molecular cloning and identification of a receptor-type protein tyrosine phosphatase, IA-2, from human insulinoma. *DNA Cell Biol* 13:505–514, 1994
12. Leslie RD, Atkinson MA, Notkins AL: Autoantigens IA-2 and GAD in type I (insulin-dependent) diabetes. *Diabetologia* 42:3–14, 1999
13. Zhang B, Lan MS, Notkins AL: Autoantibodies to IA-2 in IDDM: location of major antigenic determinants. *Diabetes* 46:40–43, 1997
14. Xie H, Zhang B, Matsumoto Y, Li Q, Notkins AL, Lan MS: Autoantibodies to IA-2 and IA-2 beta in insulin-dependent diabetes mellitus recognize conformational epitopes: location of the 37- and 40-kDa fragments determined. *J Immunol* 159:3662–3667, 1997
15. Xie J, Zhang B, Lan MS, Notkins AL: Genomic structure and promoter sequence of the insulin-dependent diabetes mellitus autoantigen, IA-2 (PTPRN). *Genomics* 54:338–343, 1998
16. Orita M, Suzuki Y, Sekiya T, Hayashi K: Rapid and sensitive detection of point mutations and DNA polymorphisms using the polymerase chain reaction. *Genomics* 5:874–879., 1989
17. Jurka J, Milosavljevic A: Reconstruction and analysis of human Alu genes. *J Mol Evol* 32:105–121, 1991
18. Yoshiuchi I, Shingu R, Nakajima H, Hamaguchi T, Horikawa Y, Yamasaki T, Oue T, Ono A, Miyagawa JI, Namba M, Hanafusa T, Matsuzawa Y: Mutation/polymorphism scanning of glucose-6-phosphatase gene promoter in noninsulin-dependent diabetes mellitus patients. *J Clin Endocrinol Metab* 83:1016–1019, 1998
19. Macfarlane WM, Frayling TM, Ellard S, Evans JC, Allen LI, Bulman MP, Ayres S, Shepherd M, Clark P, Millward A, Demaine A, Wilkin T, Docherty K, Hattersley AT: Missense mutations in the insulin promoter factor-1 gene predispose to type 2 diabetes. *J Clin Invest* 104:R33–R39, 1999
20. Hani EH, Stoffers DA, Chevre JC, Durand E, Stanojevic V, Dina C, Habener JF, Froguel P: Defective mutations in the insulin promoter factor-1 (IPF-1) gene in late-onset type 2 diabetes mellitus. *J Clin Invest* 104:R41–R48, 1999
21. Lan MS, Wasserfall C, Maclaren NK, Notkins AL: IA-2, a transmembrane protein of the protein tyrosine phosphatase family, is a major autoantigen in insulin-dependent diabetes mellitus. *Proc Natl Acad Sci U S A* 93:6367–6370, 1996
22. Marron MP, Raffel LJ, Garchon HJ, Jacob CO, Serrano-Rios M, Martinez Larrad MT, Teng WP, Park Y, Zhang ZX, Goldstein DR, Tao YW, Beaurain G, Bach JF, Huang HS, Luo DF, Zeidler A, Rotter JI, Yang MC, Modilevsky T, Maclaren NK, She JX: Insulin-dependent diabetes mellitus (IDDM) is associated with CTLA4 polymorphisms in multiple ethnic groups. *Hum Mol Genet* 6:1275–1282., 1997